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# Enzastaurin, an inhibitor of PKC beta, Enhances Antiangiogenic Effects and Cytotoxicity of Radiation against Endothelial Cells.

Aaron C. Spalding

*University of Michigan Medical School*

Benjamin David Zeitlin

*University of the Pacific, bzeitlin@pacific.edu*

Kari Wilder-Romans

*University of Michigan Medical School, kariwi@umich.edu*

Mary Ellen Davis

*University of Michigan Medical School, marydav@umich.edu*

Jacques E. Nör

*University of Michigan School of Dentistry, jenor@umich.edu*

*See next page for additional authors*

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**Authors**

Aaron C. Spalding, Benjamin David Zeitlin, Kari Wilder-Romans, Mary Ellen Davis, Jacques E. Nör, Theodore S. Lawrence, and Edgar Ben-Josef

## Enzastaurin, an inhibitor of PKC $\beta$ , Enhances Antiangiogenic Effects and Cytotoxicity of Radiation against Endothelial Cells<sup>1,2</sup>

Aaron C. Spalding\*, Benjamin D. Zeitlin<sup>†</sup>, Kari Wilder-Romans\*, Mary E. Davis\*, Jacques E. Nor<sup>†</sup>, Theodore S. Lawrence\* and Edgar Ben-Josef\*

\*The University of Michigan Medical School, Department of Radiation Oncology, Ann Arbor, MI 48109-0010, USA;

<sup>†</sup>The University of Michigan Dental School, Department of Cariology, Restorative Sciences, and Endodontics, Ann Arbor, MI 48109-0010, USA

### Abstract

**PURPOSE:** Angiogenesis plays an important role in pancreas cancer pathobiology. Pancreatic tumor cells secrete vascular endothelial growth factor (VEGF), activating endothelial cell protein kinase C beta (PKC $\beta$ ) that phosphorylates GSK3 $\beta$  to suppress apoptosis and promote endothelial cell proliferation and microvessel formation. We used Enzastaurin (Enz) to test the hypothesis that inhibition of PKC $\beta$  results in radiosensitization of endothelial cells in culture and *in vivo*. **MATERIALS/METHODS:** We measured PKC $\beta$  phosphorylation, VEGF pathway signaling, colony formation, and capillary sprout formation in primary human dermal microvessel endothelial cells (HDMECs) after Enz or radiation (RT) treatment. Microvessel density and tumor volume of human pancreatic cancer xenografts in nude mice were measured after treatment with Enz, RT, or both. **RESULTS:** Enz inhibited PKC $\beta$  and radiosensitized HDMEC with an enhancement ratio of  $1.31 \pm 0.05$ . Enz combined with RT reduced HDMEC capillary sprouting to a greater extent than either agent alone. Enz prevented radiation-induced GSK3 $\beta$  phosphorylation of serine 9 while having no direct effect on VEGFR phosphorylation. Treatment of xenografts with Enz and radiation produced greater reductions in microvessel density than either treatment alone. The reduction in microvessel density corresponded with increased tumor growth delay. **CONCLUSIONS:** Enz-induced PKC $\beta$  inhibition radiosensitizes human endothelial cells and enhances the antiangiogenic effects of RT. The combination of Enz and RT reduced microvessel density and resulted in increased growth delay in pancreatic cancer xenografts, without increase in toxicity. These results provide the rationale for combining PKC $\beta$  inhibition with radiation and further investigating such regimens in pancreatic cancer.

*Translational Oncology (2008) 1, 195–201*

### Introduction

Aberrant activation of protein kinase C $\beta$  (PKC $\beta$ ), an intracellular serine/threonine kinase, promotes endothelial cell proliferation and tumor-directed angiogenesis [1]. Tumor cells secrete vascular endothelial growth factor (VEGF) that binds to VEGFR2 on endothelial cells, resulting in the activation of PKC $\beta$  by phosphorylation at threonine 500 [1]. Active PKC $\beta$  leads to increased survival and proliferation signals, such as phosphorylation of GSK3 $\beta$  at serine 9 [2–4]. Thus, PKC $\beta$  inhibition could prevent tumor recruitment of endothelial cells and increase the effect of agents that cause endothelial cell death.

Pancreatic cancers have high microvessel density that correlates with shorter overall survival time [5], higher rates of liver metastasis, and worse prognosis [6]. Paradoxically, the microvessel density does

not lead to higher perfusion, as the pathologic angiogenesis is associated with increased vascular permeability. The resulting high

Address all correspondence to: Aaron C. Spalding, MD, PhD, Division of Radiation Oncology, Department of Radiologic Sciences, St. Jude Children's Research Hospital, 332 North Lauderdale Street, Memphis, TN 38105-2794.  
E-mail: acspalding1@gmail.com

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interstitial pressure and hypoxia [7] may contribute to the clinically observed radioresistance. Pancreatic cancers also express PKC $\beta$  at higher levels compared with surrounding tissue [8]. Interrupting tumor-mediated recruitment of blood vessels could reverse the hyperpermeable state of pancreatic tumor blood supply, and the restoration of normoxia could enhance the cytotoxic effects of radiation, providing rationale for the inhibition of PKC $\beta$  concurrent with radiation in pancreatic tumors.

Enzastaurin (Enz) is a potent and selective inhibitor of PKC $\beta$  with antiproliferative activity [inhibitory concentration of 50% (IC<sub>50</sub>) ~6 nM]. Enz suppresses VEGF-induced angiogenesis in the rat corneal micropocket assay, decreases microvessel density, and prevents VEGF secretion from human tumor cell xenografts in nude mice [9]. Prolonged courses of Enz increase chemotherapy or radiation tumor growth delay of glioma, breast, and small cell lung cancer xenografts [10]. We demonstrated that inhibition of PKC $\beta$  with enzastaurin provides modest radiosensitization of pancreatic cancer cells in culture, with increased magnitude of radiosensitization of pancreatic cancer cell xenografts [11]. Enz has been well tolerated in phase 1 and 2 clinical trials, both as monotherapy and in combination with chemotherapy [12]. However, Enz and radiation have not been combined in a prospective clinical trial, and preclinical studies examining their interaction could lead to a novel trial.

Therefore, we tested the hypothesis that inhibition of PKC $\beta$  with Enz would radiosensitize endothelial cells and would enhance the antiangiogenic effects of radiation. We first tested whether Enz could inhibit PKC $\beta$  in primary endothelial cells at concentrations similar to those attainable in patients and determined the specificity of Enz. We then used an *in vitro* model of endothelial cell sprouting to assess the effect of Enz and radiation on precursors to intact vasculature. Finally, we tested our hypothesis *in vivo* using nude mice bearing pancreatic cancer cell xenografts treated with radiation alone, Enz alone, or the combination, using tumor size and microvessel density as end points to determine efficacy.

## Materials and Methods

### Cell Lines

Primary human dermal endothelial cells (HDMECs) were obtained from Clonetics (East Rutherford, NJ) and were maintained in EGM-2MV supplemented with 50 ng/ml rhVEGF<sub>165</sub> as per the manufacturer's instructions. Panc-1 and BxPC3 human pancreatic cancer cells were obtained from the American Type Culture Collection (Manassas, VA) and were maintained in Roswell Park Memorial Institute (RPMI) medium containing 10% bovine serum in an atmosphere of 93% air and 7% carbon dioxide.

### Colony Formation Assays

After Enz treatment or irradiation, HDMECs were trypsinized, counted, and plated at clonal densities. Fourteen days later, cells were fixed and stained with crystal violet, as previously published by others [13]. Colony counting was done using an automated counter. The mean inactivation dose (MID) [14] was calculated for control and each dose of Enz, and the enhancement ratio (ER) was calculated as the MID in the control curve divided by the MID in the Enz curve. The ERs shown are the average of three independent experiments done in triplicate.

### Capillary Sprouting

To investigate the effect of Enz on the angiogenic potential of primary endothelial cells, a capillary sprouting assay was done as described previously [15]. Briefly, six-well plates were precoated with 1.5 mL/well Vitrogen 100 collagen (Angiotech BioMaterials, Palo Alto, CA). Human dermal microvessel endothelial cells ( $5 \times 10^5$ ) were added to each well and allowed to adhere overnight. Cells were treated daily with 50 ng/mL rhVEGF<sub>165</sub> in fresh medium until cells were treated as described in the figure legends. Numbers of sprouts were assessed daily with a phase microscope at an original magnification of  $\times 200$ . Six high-power fields were analyzed per well with triplicate wells per treatment, and a minimum of three independent experiments were conducted.

### Antibodies and Immunoblot Analysis

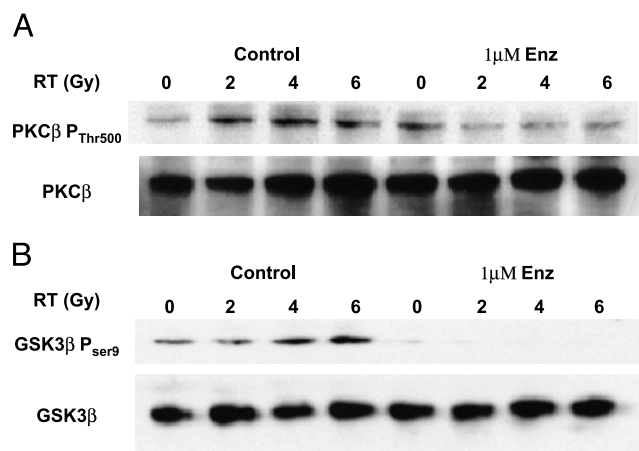
Antibodies to PKC $\beta$  (Santa Cruz Biotechnology, Santa Cruz, CA), phosphoThr500 PKC $\beta$  (Abcam, Cambridge, MA), GSK3 $\beta$  (Cell Signaling, Danvers, MA), phosphoSer21GSK3 $\alpha$ /Ser9GSK3 $\beta$  (Cell Signaling), VEGFR2 (Cell Signaling), phosphoTyr951 VEGFR2 (Cell Signaling), p42/44 mitogen-activated protein kinase (MAPK; Cell Signaling), and phosphoThr202/Tyr204 p42/44 MAPK (Cell Signaling) were used at dilutions recommended by the manufacturer. Cell lysate production with RIPA buffer and immunoblot analysis were performed using detailed protocols from Cell Signaling. Protein concentrations were determined with BCA Protein Assay (Pierce, Rockford, IL), and 10  $\mu$ g of protein was loaded in each lane. Each experiment was conducted a minimum of three independent times.

### Immunohistochemistry

We determined that five 2-Gy fractions in Panc1 xenografts produced similar tumor growth delay as ten 2-Gy fractions in BxPC3 xenografts [11]. To compare the effects of enzastaurin on biologically similar end points, we used 5 fractions in the Panc1 experiments and 10 fractions in the BxPC3 experiments. Enzastaurin was given twice daily concurrently with radiation. Xenografts were collected on the last day of treatment for each group and placed in formalin overnight followed by 70% ethanol. Samples were embedded in paraffin, and immunohistochemistry was performed with anti-CD31 for microvessel density (MVD) by the immunohistochemistry core at the University of Michigan. To determine MVD, the slides were blinded to the counter, and the number of patent vessels was scored in 10 high-powered ( $\times 400$ ) fields.

### Xenografts

Animals used in this study were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care in accordance with current regulations and standards of the United States Department of Agriculture and Department of Health and Human Services. Under an institutionally approved protocol, 4-week-old female athymic nude mice were implanted with  $5 \times 10^7$  BxPC3 or Panc1 cells subcutaneously. Tumor volume (TV) was calculated according to the following equation:  $TV = \pi/6 \times a \times b^2$ , where  $a$  and  $b$  are the longer and shorter dimensions of the tumor, respectively. When the average tumor volume achieved 100 mm<sup>3</sup>, mice were randomized to treatment groups of vehicle alone, Enz (100-mg/kg gavage twice daily) alone, radiation alone, or Enz with radiation. This dose of Enz produces similar serum concentrations to



**Figure 1.** Enzastaurin prevents PKC $\beta$  T500 phosphorylation and GSK3 $\beta$  S9 phosphorylation in irradiated primary endothelial cells. Human dermal microvessel endothelial cells were treated with vehicle or 1  $\mu$ M Enz for 24 hours and then exposed to 0, 2, 4, or 6 Gy. (A) Western blot analysis for total or phosphoT500 PKC $\beta$  was performed. (B) Western blot analysis for total (bottom row) or phosphoS9 (top row) GSK3 $\beta$  was performed.

those achieved in patients from phase 1 trials, approximately 1  $\mu$ M [16]. Radiation was given 3 to 4 hours after Enz, corresponding to the maximum amount of GSK3 $\beta$  dephosphorylation seen in mouse xenografts [16]. Data are expressed as the ratio of TV at varying times after treatment compared to the day of irradiation (Day 0). The absolute and normalized growth delays were calculated to compare the efficacy of each regimen. Absolute growth delay is defined as the time in days for tumors in the treatment arms to quadruple their volume minus the time in days for the tumors in the untreated control group to reach the same size. Normalized growth delay is defined as the time for tumors in groups treated with a combined regimen to quadruple their volume minus the time to reach the same size in mice treated with Enz alone [17].

### Irradiation

Cells or xenografts were irradiated using a Phillips 250 orthovoltage unit (Phillips, Bothell, WA) at approximately 2 Gy/min for cells or 1.4 Gy/min for mice in the Irradiation Core of the University of Michigan Cancer Center. Dosimetry was carried out using an ionization chamber connected to an electrometer system, which is directly traceable to a National Institute of Standards and Technology calibration. Mice were anesthetized with a mixture of 60-mg/kg ketamine and 3-mg/kg xylazine and were positioned such that the apex of each flank tumor was at the center of a 2.4-cm aperture in the secondary collimator and irradiated with the rest of the mouse being shielded from radiation.

### Statistical Analysis

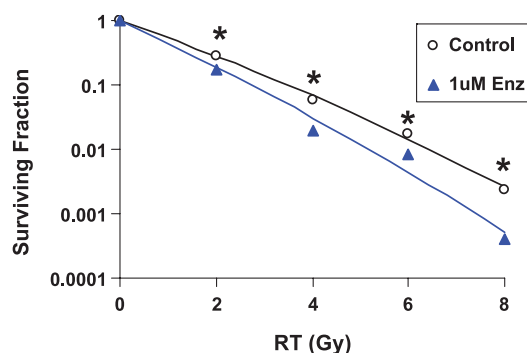
Differences between groups in microvessel density, tumor size, or mean growth delay were tested for significance with an unpaired, 2-tailed, Student's *t* test. Analysis of variance was used for simultaneous multiple comparisons across more than two treatment arms. The xenograft studies were powered at 80% to detect a 30% difference in absolute growth delay between the combination treatment arm and control.

## Results

We tested the effects of radiation and Enz on PKC $\beta$  activity by immunoblot analysis for phosphorylated PKC $\beta$ . Active PKC $\beta$  can phosphorylate GSK3 $\beta$  at serine 9, and others have shown that radiation induces GSK3 $\beta$  serine 9 phosphorylation in endothelial cells [18]. We tested whether radiation or Enz treatment affected GSK3 $\beta$  serine 9 phosphorylation. Primary human endothelial cells were treated with radiation with or without pretreatment with 1  $\mu$ M Enz for 24 hours. Radiation induced phosphorylation of threonine 500, consistent with activation of PKC $\beta$  in a dose-dependent manner (Figure 1A). Similarly, radiation induced the phosphorylation of GSK3 $\beta$  serine 9 (Figure 1B). Pretreatment for 24 hours with 1  $\mu$ M Enz prevented radiation-induced PKC $\beta$  T500 phosphorylation and GSK3 $\beta$  serine 9 phosphorylation. Total levels of PKC $\beta$  and GSK3 $\beta$  were unchanged. In primary endothelial cells grown in culture, Enz inhibited radiation-induced PKC $\beta$  activation and GSK3 $\beta$  serine 9 phosphorylation.

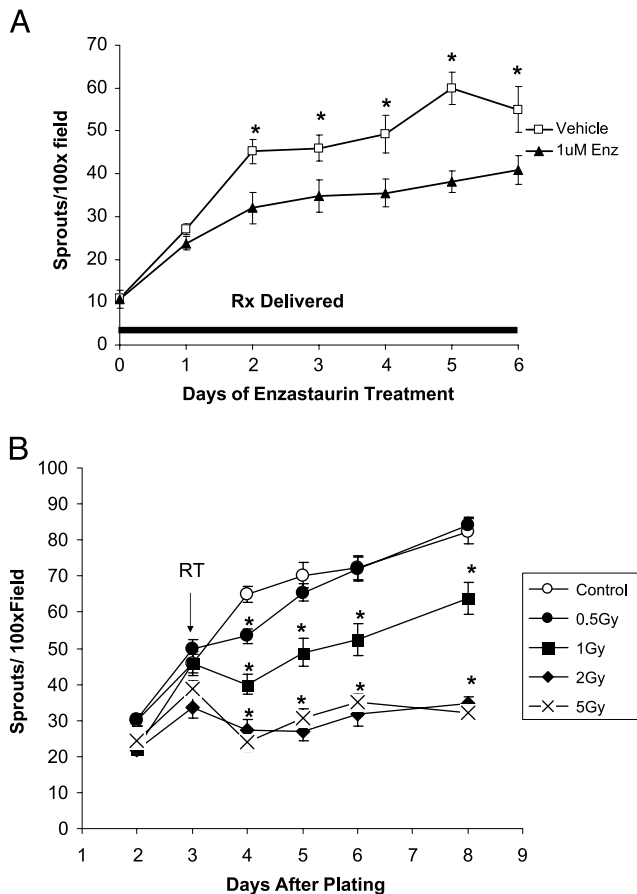
We next assessed the effect of Enz on radiation cytotoxicity in endothelial cells by colony formation in culture. Primary human endothelial cells in log-phase growth were treated with incremental doses of radiation with or without pretreatment with 1  $\mu$ M Enz for 24 hours (Figure 2A), resulting in decreased clonogenic survival with Enz ( $P < .05$ ). Enz produced an ER of  $1.31 \pm 0.05$  ( $n = 3$ ) in primary endothelial cells. These data indicate that Enz modestly radiosensitizes primary endothelial cells grown in culture.

We next conducted experiments to determine the ability of Enz, radiation, or the combination to influence primary endothelial cell formation of capillary sprouts in culture. Human dermal microvessel endothelial cells treated with daily Enz alone had significantly reduced capillary sprout formation (Figure 3A) relative to vehicle-treated wells after 2 days ( $32 \pm 4$  vs  $45 \pm 3$  sprouts,  $P < .01$ ) and persisted through 6 days of treatment ( $38 \pm 3$  vs  $55 \pm 4$  sprouts,  $P < .01$ ,  $n = 3$ ). We conducted a dose-response experiment with single-fraction radiation to determine the IC<sub>50</sub> dose of radiation to combine with Enz (Figure 3B), as radiation has not been previously used in this model. Human dermal microvessel endothelial cells treated with 0.5 Gy were able to achieve maximum sprout formation at day 8 ( $84 \pm 2$  sprouts). Primary endothelial cells treated with radiation had reduced capillary sprout formation relative to control ( $64 \pm 4$  for 1 Gy vs  $35 \pm 2$  for 2 Gy vs  $32 \pm 3$  for 5 Gy vs  $82 \pm 4$  sprouts for control, respectively,



**Figure 2.** Brief course of Enzastaurin increases radiation cytotoxicity of primary endothelial cells. Human dermal microvessel endothelial cells were treated with vehicle or 1  $\mu$ M Enz for 24 hours and then exposed to 0, 2, 4, 6 or 8 Gy. Colony formation was assessed per the Materials and Methods section.





**Figure 3.** Prolonged enzastaurin treatment or higher doses of RT reduce endothelial cell capillary sprout formation. (A) Human dermal microvessel endothelial cells formation of capillary sprouts on a collagen matrix treated with vehicle (open circles) or daily 1  $\mu$ M Enz (black triangles). (B) Human dermal microvessel endothelial cells formation of capillary sprouts on a collagen matrix treated with vehicle (open circles), 0.5 Gy (black circles), 1 Gy (black squares), 2 Gy (black diamonds), or 5 Gy (black X). The values displayed represent mean  $\pm$  SE of two independent experiments performed in triplicate. \* $P < .05$  between control and indicated radiation dose.

$P < .001$  for all groups *vs* control,  $n = 3$ ). Both 2 and 5 Gy virtually eliminated the ability of HDMEC to form capillary sprouts during the 8 days. We proceeded with a single 1-Gy fraction to combine with Enz, because either radioprotection or radiosensitization could be demonstrated with this dose.

We also determined the effect of 1 Gy with Enz on VEGFR signaling. As shown in Figure 4A, 1  $\mu$ M Enz inhibited both PKC $\beta$  and GSK $\beta$  phosphorylation. To assess the specificity of Enz, we also assessed VEGFR2, the receptor for VEGF, and p42/44 MAPK, a parallel intracellular kinase important VEGF-mediated for angiogenesis [19,20], levels by Western blot analysis. As shown in Figure 4B, Enz treatment did not change VEGFR or p42/44 MAPK phosphorylation, suggesting that Enz under these conditions does not change VEGFR activation or p42/44 MAPK phosphorylation.

As shown in Figure 4, C and D, the combination of 1  $\mu$ M Enz with 1-Gy radiation resulted in greater reduction in capillary sprout formation than either agent alone ( $34 \pm 4$  sprouts with combination *vs*  $93 \pm 9$  for Enz *vs*  $73 \pm 4$  for 1 Gy *vs*  $110 \pm 4$  for control,  $P < .001$  for

all groups *vs* combination,  $n = 3$ ). Similar results were achieved with 0.3  $\mu$ M Enz and 1 Gy (Figure W1). These experiments demonstrate that Enz and radiation in combination inhibit capillary sprout formation on a collagen matrix to a greater extent than either agent alone.

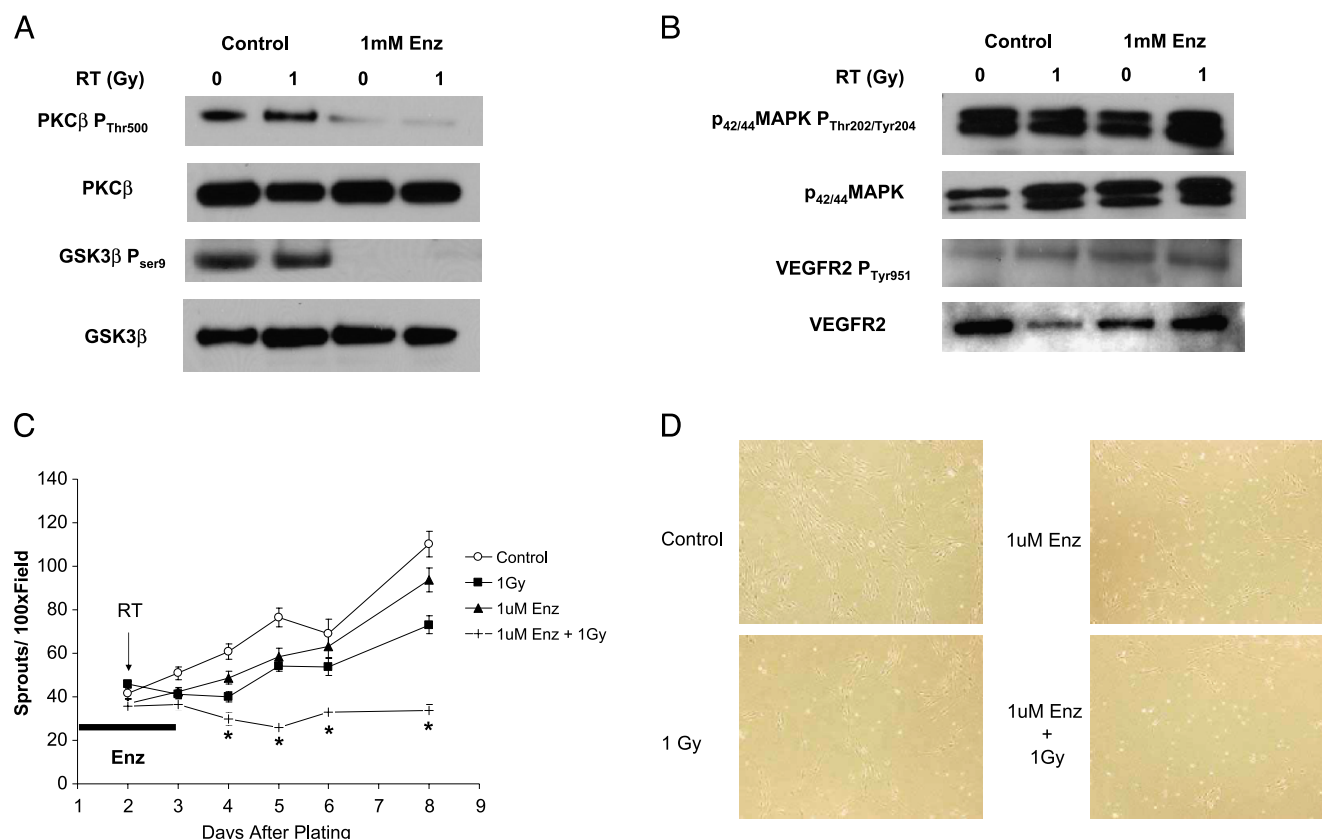
We next conducted experiments to determine the effect of enzastaurin on pancreatic cancer cells on endothelial cell VEGFR2 signaling, because pancreatic cancer cells can secrete VEGF to promote angiogenesis [21–23]. Conditioned medium from BxPC3 and Panc1 cells induced VEGFR2 phosphorylation, which was not affected by Enz (Figure 5). However, Enz did prevent the GSK3 $\beta$  phosphorylation that occurred after exposure to conditioned medium. These results are consistent with Enz preventing pancreatic cancer cell angiogenic signals downstream of VEGFR2.

Given the results with primary endothelial cells in culture, we sought to determine the ability of Enz, radiation, and the combination to reduce microvessel density of pancreatic cancer cell xenografts. Nude mice bearing either Panc1 or BxPC3 xenografts were treated with vehicle, Enz, five 2-Gy fractions to the tumor, or concurrent Enz and radiation (Figure 6). Untreated Panc1 tumors had a microvessel density of  $15 \pm 1$ , whereas BxPC3 cells had  $21 \pm 1$  vessel per high-powered field. Treatment of Panc1 and BxPC3 xenografts with Enz and radiation reduced microvessel density greater than either treatment alone (MVD  $\pm$  SD):  $3 \pm 1$  and  $8 \pm 1$  for RT with Enz,  $10 \pm 1$  and  $13 \pm 1$  for RT alone, and  $8 \pm 1$  and  $19 \pm 8$  for Enz alone ( $P < .01$  between combination *vs* other arms), respectively. There was no difference in TUNEL-positive cells between any of the treatment arms (Figure W2). These xenograft experiments are consistent with the primary endothelial microvessel formation experiments demonstrating that the combination of Enz and radiation disrupt vasculature greater than either alone.

To determine whether the reductions in microvessel density resulted in prolonged tumor growth delay, a larger experiment was conducted with BxPC3 xenograft bearing mice. Mice were treated with vehicle alone, Enz, five 2-Gy fractions to the tumor, or Enz and five 2-Gy fractions (Figure 6C). The combination of Enz with radiation had decreased tumor volume compared to the other treatment arms by day 15 ( $248 \pm 61$  mm<sup>3</sup> for control *vs*  $346 \pm 43$  mm<sup>3</sup> for Enz alone *vs*  $257 \pm 41$  for radiation alone *vs*  $160 \pm 30$  for combination,  $P < .03$  for combination *vs* other groups,  $n = 16$  tumors). The combination arm resulted in prolonged absolute tumor growth delay (5.6 days), whereas those treated with either Enz (0.1 days) or radiation alone (0.3 days) did not demonstrate significant growth delay. This resulted in a normalized growth delay of 5.5 days. Thus, in this model of aggressive pancreatic cancer, neither Enz nor radiation alone produced effective tumor growth delay, whereas the combination had efficacy.

## Discussion

We tested the hypothesis that inhibition of PKC $\beta$  with Enz potentiates the antivascular and cytotoxic effects of radiation on endothelial cells in culture and *in vivo*. We found that Enz inhibits PKC $\beta$  and phosphorylation of its downstream target GSK $\beta$  in primary endothelial cells at concentrations similar to those achieved in patients. In a model of endothelial cell sprouting, we found that Enz and radiation together had a larger effect than either alone on precursors to intact vasculature. Pancreatic cancer cell xenografts treated with the combination of radiation and Enz had decreased microvessel density with corresponding increased tumor growth delay compared to either agent alone. Taken together, these data provide evidence that



**Figure 4.** Brief course of enzastaurin potentiates the antivascular effects of 1-Gy radiation. Human dermal microvessel endothelial cells were treated with vehicle or 1  $\mu$ M Enz for 24 hours and then exposed to 0 or 1 Gy. Western blot analysis of phosphorylated and total PKC $\beta$  and GSK3 $\beta$  (A) or p42/44 MAPK and VEGFR2 (B) was performed. (C) Human dermal microvessel endothelial cells formation of capillary sprouts on a collagen matrix treated with vehicle (open circles), 1 Gy (black squares), 1  $\mu$ M Enz for 48 hours (black triangles), or 1 Gy in the middle of 1  $\mu$ M Enz for 48 hours (black +). Mean values of three independent experiments performed in triplicate  $\pm$  SEM are displayed. \* $P$  < .05 between combination arm and control or single agent arms. (D) Representative images of HDMEC at day 7 from B.

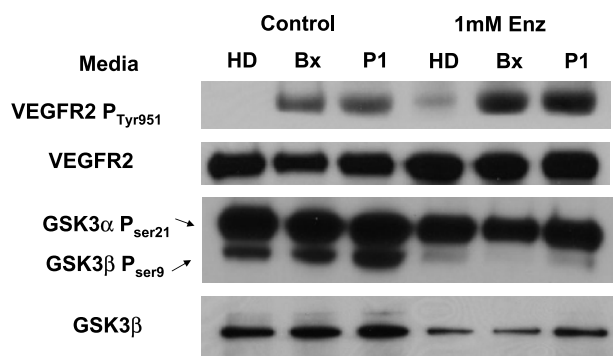
inhibition of PKC $\beta$  with Enz radiosensitizes endothelial cells in culture and *in vivo*, suggesting this approach may improve radiation response of tumors through antivascular effects.

Protein kinase C inhibition alone or in combination with radiation has efficacy in preclinical models of cancer. Protein kinase C  $\beta$  promotes colon [24] and breast [25] cancer cell cycle progression. Tar-

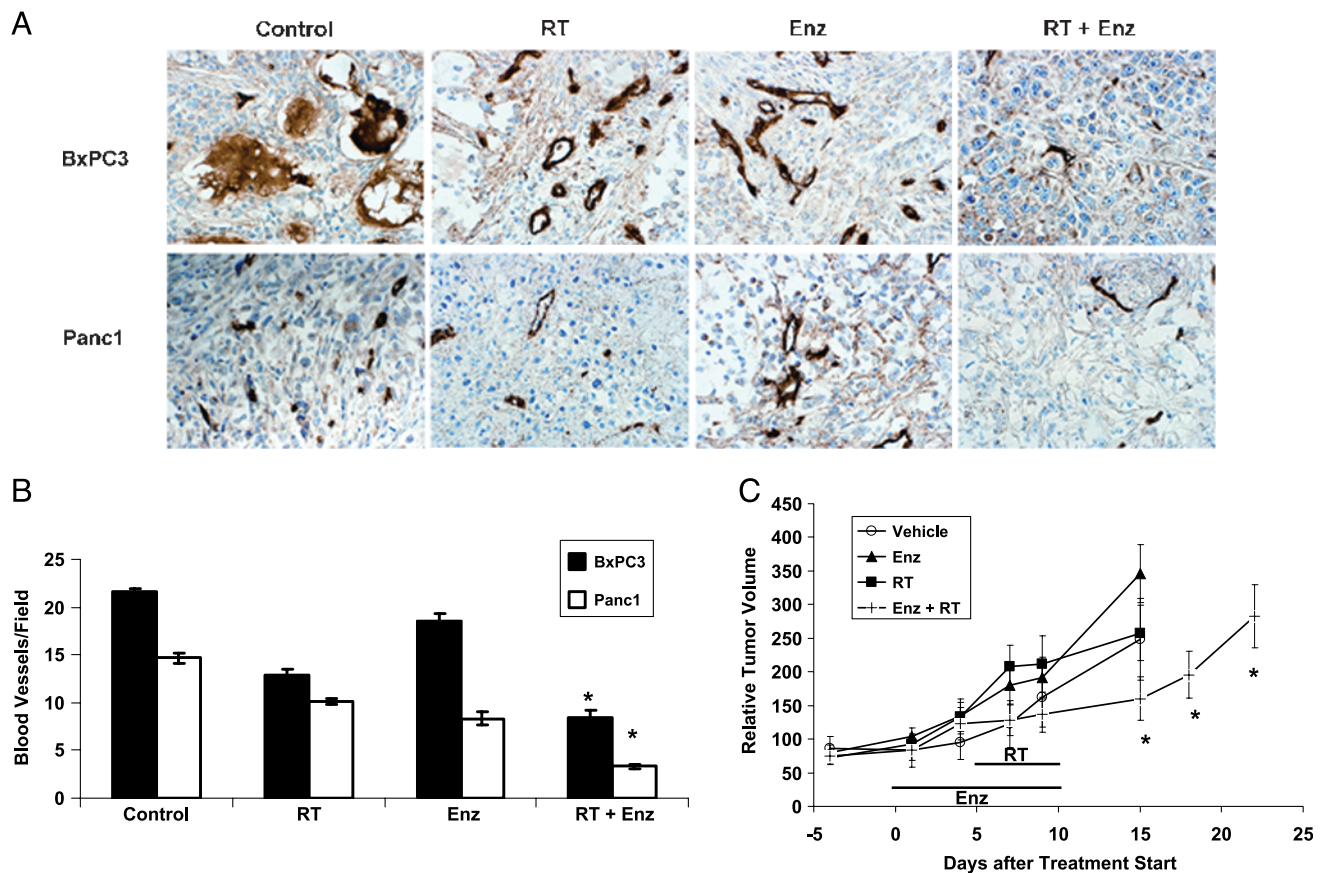
geted silencing of PKC $\beta$  or pharmacologic inhibition has been shown to inhibit proliferation in culture of colon, glioma, breast, and ovarian cancer cells. Others have shown that pharmacologic agents that inhibit PKC isoforms can radiosensitize cancer cells in culture and *in vivo* [4,26]. Our results also are consistent with and build on these previously published works by focusing on the effect of PKC $\beta$  inhibition and radiation in endothelial cells.

Tumor response to radiotherapy can be improved by targeting angiogenesis signaling pathways [27–29]. The underlying mechanisms are not fully understood, but it has been suggested that induction of VEGF by radiation [27] may contribute to radioresistance by promoting survival of endothelial cells in tumor vasculature. Other pro-angiogenic growth factors, including fibroblast growth factor and platelet derived growth factor [30,31], are also known to be up-regulated by radiation and may play similar role. In addition, tumor oxygenation, critical to the efficacy of radiation, has been shown to improve during VEGFR2 blockade [32]. Enz is a potent and selective inhibitor of PKC $\beta$ , a predominant mediator of VEGF-induced endothelial cell proliferation and survival [33,34]. Thus, it is possible that Enz could enhance radiation cytotoxicity of tumors through its antivascular effects.

A body of evidence points to the importance of PKC $\beta$ -dependent angiogenesis in pancreas cancer. Protein kinase C  $\beta$  is overexpressed in pancreatic cancer relative to surrounding stroma [8], and VEGFR activation leads to PKC $\beta$ -dependent endothelial cell proliferation [1].



**Figure 5.** Effect of pancreatic cancer secretions on endothelial cell VEGFR signaling. Human dermal microvessel endothelial cells were treated with control medium (HD), medium from BxPC3 cells (Bx), or medium from Panc1 cells (P1), with or without enzastaurin. Western blot analysis of phosphorylated and total VEGFR2 and GSK3 $\beta$  was performed.



**Figure 6.** Enzastaurin potentiates radiation-induced reductions in pancreatic tumor xenograft microvessel density and prolongs radiation tumor growth delay *in vivo*. (A) Nude mice bearing BxPC3 or Panc1 xenografts were treated with vehicle, Enz 100 mg/kg twice daily, 2 Gy once daily, or Enz and radiation. At least four tumors were collected immediately after the last treatment rendered and were stained for CD31. Shown are representative sections from each treatment arm. (B) Quantitation of the microvessel density for the treatment groups shown in A. (C) Nude mice bearing BxPC3 xenografts were treated for 5 days with vehicle (open circles), Enz 100 mg/kg twice daily (black triangles), 2 Gy once daily (black squares), or Enz and radiation (black +). \* $P < .05$  between combination and single agent arms by analysis of variance.

Both VEGF [21–23] and VEGF receptors [35] are overexpressed in pancreatic cancer. Vascular endothelial growth factor promotes pancreatic cancer growth through a paracrine and/or autocrine mechanism [22,35,36] and that VEGFR promotes migration [37], invasion, and mesenchymal transition [38] in pancreatic carcinoma cell lines. Finally, high VEGF expression correlates with microvessel density and with poor prognosis [21–23] in patients.

There are limitations to our study. We have attempted to study the effects of Enz and radiation on endothelial cell survival and ability to form blood vessels. The sprouting of endothelial cells on a bed of collagen occurs for several days, allowing for migration and proliferation contributions to angiogenesis. This model is ideal for determining doses and schedules of radiation and systemic agents, but nonetheless, it must be tested *in vivo*. Our xenograft model of pancreatic cancer has the limitations of mouse vasculature with human tumor cells. It is likely that tumors that arise *de novo* in patients have different mechanisms for endothelial cell recruitment. Despite these limitations, our studies give merit to inhibition of PKC $\beta$ -dependent angiogenesis with radiation in the treatment of cancer.

In summary, our results support the use of Enz with radiation to target endothelium. Tumor-induced angiogenesis requires activation of the PKCs, particularly PKC $\beta$  [39], and Enz is a potent and selective inhibitor of PKC $\beta$ . Thus, the rationale for antiangiogenic therapy

through PKC $\beta$  inhibition in pancreas cancer is solid. A natural extension of these studies would be to investigate the effects of Enz, radiation, and cytotoxic chemotherapeutic agents used in the treatment of pancreatic cancer such as gemcitabine and 5-fluorouracil on endothelial cells. Our results suggest that regimens combining PKC $\beta$  inhibition with radiation might be worthwhile investigating in clinical trials.

## Acknowledgments

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## Conflicts of Interest Notification

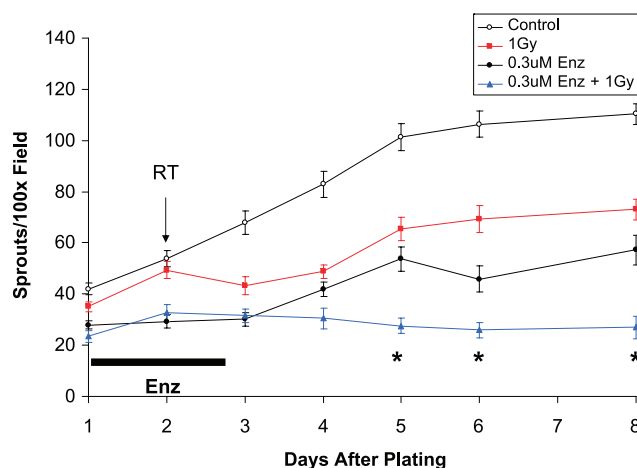
Lilly, the manufacturer of enzastaurin, provided the compound we used in the manuscript to inhibit PKC $\beta$ . Lilly had no role or input in the experimental design, data analysis, or manuscript writing. E.B.-J. has previously received research support from Lilly.

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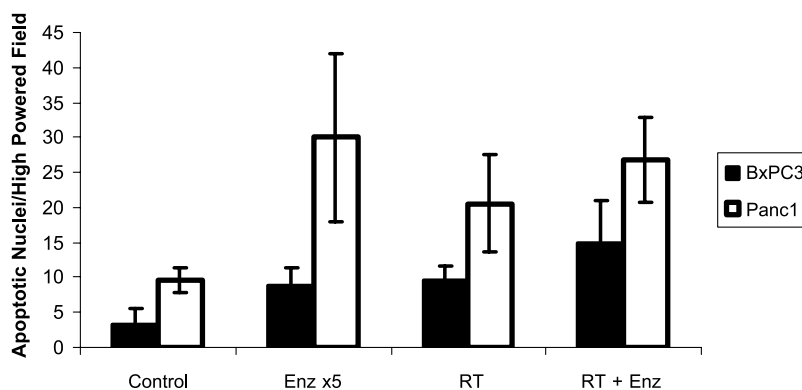
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**Figure W1.** Human dermal microvessel endothelial cell formation of capillary sprouts on a collagen matrix treated with vehicle (open circles), 1 Gy (red squares), 0.3  $\mu$ M Enz for 48 hours (black circles), or 1 Gy in the middle of 0.3 mM Enz for 48 hours (blue triangles). Mean values of three independent experiments performed in triplicate  $\pm$  SEM are displayed. \* $P < .05$  between combination arm and control or single agent arms.



**Figure W2.** Nude mice bearing BxPC3 or Panc1 xenografts were treated with vehicle, Enz 100 mg/kg twice daily, 2 Gy once daily, or Enz and radiation. At least four tumors were collected immediately after the last treatment rendered and were stained for TUNEL.  $P =$  NS between combination arm and individual treatment arms.